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Dexamethasone induces caspase activation in murine osteoblastic MC3T3-E1 cells

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Abstract

Glucocorticoids are widely used as anti-inflammatory and chemotherapeutic agents. However, prolonged use of glucocorticoids leads to osteoporosis. This study was designed to examine the mechanism of dexamethasone (DEX)-induced apoptosis in murine osteoblastic MC3T3-E1 cells. Total RNA was extracted from MC3T3-E1 cells treated with 10^{-7} M DEX for 6 h. DEX exerted a variety of effects on apoptotic gene expression in osteoblasts. Ribonuclease protection assays (RPA) revealed that DEX upregulated mRNA levels of caspases-1, -3, -6, -8, -11, -12, and bcl-X_L. Western blot analysis showed enhanced processing of these caspases, with the appearance of their activated enzymes 8 h after DEX treatment. In addition, DEX also induced the activation of caspase-9. DEX elevated the levels of cleaved poly(ADP-ribose) polymerase and lamin A, a caspase-3 and a caspase-6 substrate, respectively. Expression of bcl-X_L protein level was upregulated by DEX. Cytochrome *c* release was detected in the cytosol of DEX-treated cells. Furthermore, caspase-3 enzyme activity was elevated by 2-fold after DEX treatment for 7 h. Finally, early apoptotic cells were detected in cells treated with DEX for 3 h. Our results demonstrate that DEX-induced apoptosis involves gene activation of a number of caspases.

Keywords: Dexamethasone; Caspase; Apoptosis; Osteoblast

1. Introduction

Apoptosis, or programmed cell death, is crucial for a number of processes including development, tissue homeostasis, viral infection, and cancer [1–3]. Apoptosis is characterized by cell shrinkage, membrane blebbing, chromatin condensation, and nuclear DNA degradation. The mechanism of apoptosis involves a cascade of activation of initiator and effector caspases. So far, 14 caspases have been identified. These enzymes are involved in proteolytic cleavage that leads to cell disassembly [4–7].

Apoptosis plays a pivotal role in the regulation of bone turnover [8,9]. In fact, Jilka et al. [10] demonstrated that apoptosis is the main fate for most osteoblasts. Because bone diseases such as osteoporosis can be caused by a relatively decreased activity or decreased numbers of osteoblasts, prevention of osteoblast apoptosis can be an extremely important clinical goal.

Glucocorticoids (GC) are widely used as chemotherapeutic and anti-inflammatory agents. However, prolonged administration of glucocorticoids is one of the leading causes of osteoporosis [11]. It is clear that glucocorticoids have detrimental effects on the proliferation and function of osteoblasts. For example, dexamethasone (DEX), a synthetic GC hormone, has been shown to inhibit the synthesis of fibronectin and collagen and to stimulate collagenase synthesis [12–15]. All of these effects lead to decreased bone formation.

Several lines of evidence indicate that glucocorticoid induces apoptosis in bone. Weinstein et al. [16] demonstrated that prednisone increases the rate of apoptosis in both osteoblasts and osteocytes in adult mice. In addition, Gohel et al. [17] reported that corticosterone induces apoptosis in rat and mouse osteoblasts by decreasing bcl_2/Bax ratio.

Although glucocorticoid has been shown to induce apoptosis in osteoblasts, the exact mechanism and the involvement of caspases in glucocorticoid-induced apoptosis in osteoblasts are not clearly understood. In this study, we report that DEX induced apoptosis in murine osteoblastic MC3T3-E1 cells by activating caspases-1, -3, -6, -8, -9, -11, and -12. In addition, DEX also upregulated the expression of bcl-X_L.

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2. Materials and methods

2.1. Cell culture and materials

Murine osteoblastic MC3T3-E1 cells were cultured in α-modified MEM (Sigma Chemical Co., St. Louis, MO) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 μg/ml gentamicin at 37 °C in a humidified atmosphere of 5% CO_2. Cells were serially subcultured by treatment with trypsin and were used for experimentation between passages 5 and 15. DEX was obtained from Calbiochem (San Diego, CA) and was used at 10^{-7} M throughout the study. α-[^{32}P]UTP was obtained from ICN (Costa Mesa, CA). FBS was obtained from Biofluid Division-Biosource International (Rockville, MD). Mouse apoptosis gene templates mAPO1 and mAPO2 were obtained from BD Pharmingen (San Diego, CA). Caspase-1 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Caspase-3 and caspase-8 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-6, cleaved caspase-9, cleaved PARP and lamin A antibodies were products of Cell Signaling Technology (Beverly, MA). Rat monoclonal caspases-11 and -12 antibodies were generous gifts from Dr. Junying Yuan of Harvard Medical School. Mouse monoclonal actin antibody (clone AC-40) was purchased from Sigma. Cytochrome *c* and COX-4 antibodies were products of BD Pharmingen and Molecular Probes (Eugene, OR), respectively.

2.2. Ribonuclease protection assay

MC3T3-E1 cells were seeded into 100 mm dishes containing α-MEM supplemented with 10% FBS. Near confluent cells were changed to α-MEM containing 0.5% FBS for 16 h before the addition of 10^{-7} M DEX for 6 h. Total RNA was extracted using acid guanidinium thiocyanate-phenol-$CHCl_3$ extraction method. Ribonuclease protection assay (RPA) was performed according to manufacturer's instructions (BD Pharmingen). Briefly, murine apoptosis template set mAPO-1 (caspases-1, -2, -3, -6, -7, -8, -11, -12, and -14) and mAPO-2 (bcl-w, bfl1, bcl-x, bak, bax, bcl-2, bad) were labeled with [α-^{32}P]UTP. RNA (10 μg) and labeled probes were hybridized and the protected mRNAs were resolved on a 5% denaturing polyacrylamide gel. Intensity of the band was scanned by a Digital Imaging System (Alpha Innotech Corp., San Leandro, CA).

2.3. Western blot analysis

Near confluent MC3T3-E1 cells in 100 mm dishes were incubated with α-MEM containing 0.5% FBS for 16 h before treatment with 10^{-7} M DEX for 2, 4, or 8 h. Cells were lysed in a buffer containing 50 mM Pipes, pH 6.5, 2 mM EDTA, 0.1% Chaps, 20 μg/ml leupeptin, 10 μg/ml each of aprotinin and pepstatin, and 5 mM dithiothreitol. Protein concentration was determined by Coomassie dye binding assay (BioRad, Hercules, CA). Aliquots of 50 μg of lysates were electrophoresed on 12% SDS-PAGE and transferred to nitrocellulose membranes. Western blot analysis was carried out with optimal dilutions of antibodies against caspases-1, -3, -6, -8, -9, -11, -12, cleaved PARP, lamin A, and actin. Appropriate secondary antibodies conjugated to horseradish peroxidase were then added for 1.5 h. Antigen–antibody complex was detected using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Intensity of the band was subjected to image analysis.

2.4. Caspase-3-like activity assay

Caspase-3-like activity was assayed using CaspaTag 3 activity kit (Serologicals Corp., Norcross, GA). Briefly, cells in 100 mm plates were treated with 10^{-7} M DEX for 7 h. After trypsinization, cells were resuspended in 300 μl of wash buffer and incubated with a working dilution of fluorochrome-labeled caspase-3 inhibitor (FAM-DEVD-FMK) for 1 h. Cells were washed three times with wash buffer, resuspended in PBS, and read in a fluorescence plate reader (excitation 485 nm, emission 535 nm). Results were expressed as fluorescence units/10^6 cells.

2.5. Apoptotic cell staining

Detection of early apoptotic cells was performed with APOPercentage Apoptosis assay kit (Biocolor Co., Belfast, Northern Ireland). Briefly, cells were seeded on gelatin-coated chamber slides and treated with 10^{-7} M DEX for 3 h. Cells were then incubated with 100 μl of dye provided in the kit for 1 h, washed, and photographed.

2.6. Cytochrome c release

Mitochondrial and cytosolic fractions were prepared from control and DEX-treated MC3T3-E1 cells for 16 h according to the protocol of Yang et al. [18]. Cytosolic fractions (20 μg) and mitochondria (5 μg) were separated on 14% SDS-PAGE and probed with cytochrome *c* and COX-4 antibodies.

2.7. Statistical analysis

Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison test to show differences between means. Data were represented as means ± S.E. $P < 0.05$ was considered significant.

3. Results

Our first set of experiments was designed to study the effect of DEX on the mRNA levels of caspases and bcl-2

family members by RPAs. Several doses (10^{-8}, 10^{-7}, and 10^{-6} M) of DEX were tested and 10^{-7} M was found to be optimal (results not shown). Treatment of MC3T3-E1 cells with 10^{-7} M DEX for 6 h upregulated the mRNA levels of caspases-1, -3, -6, -8, -11, and -12, whereas the mRNA levels of caspases-2, -7, -10, and -14 remained unchanged (Fig. 1). In addition to its effects on the aforementioned caspases, DEX also upregulated the mRNA levels of bcl-X_L while the levels of the rest of the bcl-2 family members remained the same (Fig. 2). Similar results were obtained with 3 h treatment (results not shown). In these experiments, L32 and GAPDH served as loading controls. Table 1 summarizes a quantitative analysis of the magnitude of induction for these six caspases. The induction ranged from 1.6-fold to 3.2-fold. In addition, the mRNA level of bcl-X_L was also upregulated by 3.2-fold.

To investigate the expression of caspases at the protein level, lysates were prepared from cells treated with DEX for 2, 4, or 8 h. Caspase-1 was present as 45, 36, 32 and 20 kDa bands in untreated cells. After exposure to DEX, all bands were enhanced in intensity. The smaller fragments of 36, 32, and 20 kDa represented the cleavage products of 45 kDa procaspase-1 (Fig. 3). Caspase-3 is the major effector cysteine protease in the apoptotic pathway. The activation of caspase-3 was detected with an antibody that recognized the 20 kDa fragment of the active caspase-3. Fig. 3 shows that DEX induced the active form of caspase-3 in a time-dependent manner. Caspase-6, another effector caspase, was present as a 35 kDa band in untreated cells. After DEX treatment, the 35 kDa band increased in intensity with a concomitant appearance of the activated 15 kDa fragment. Fig. 3 also demonstrates that DEX induced the activation of caspase-8, as evidenced by the appearance of the 20 kDa fragment.

Since the gene template used in our RPA studies (Fig. 1) did not contain caspase-9, Western blot analysis was carried

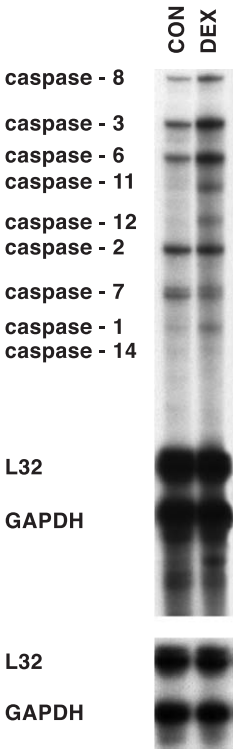

Fig. 1. Caspase mRNA expression in MC3T3-E1 cells. RNA was extracted from MC3T3-E1 cells treated with 10^{-7} M DEX for 6 h. Ribonuclease protection assay was performed with mAPO1 template set as described in Materials and methods. The specific mRNA transcripts are labeled using the nomenclature from the supplier. Lower panel shows shorter exposure of L32 and GAPDH bands. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

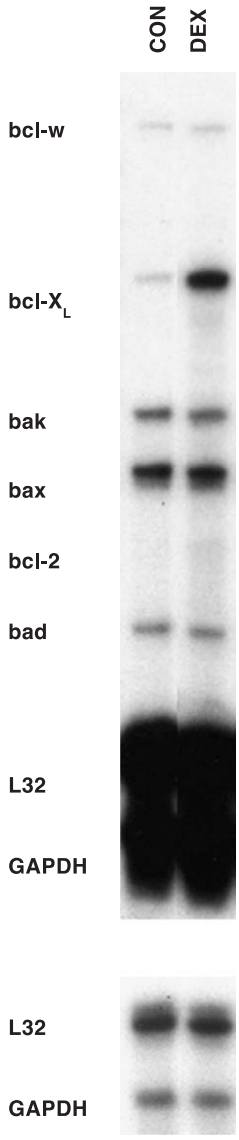

Fig. 2. Message level of bcl-2 family proteins in MC3T3-E1 cells. RNA was extracted from MC3T3-E1 cells treated with DEX for 6 h. Ribonuclease protection assay was performed with mAPO2 template set as described in Materials and methods. Lower panel shows shorter exposure of L32 and GAPDH bands.

Table 1
Fold inductions of mRNA levels

	Control	DEX	Fold
Caspase-1	0.42 ± 0.04	0.74 ± 0.06[a]	1.8
Caspase-3	0.62 ± 0.04	0.99 ± 0.04[a]	1.6
Caspase-6	0.60 ± 0.08	1.05 ± 0.02[a]	1.8
Caspase-8	0.33 ± 0.02	0.56 ± 0.05[a]	1.7
Caspase-11	0.28 ± 0.01	0.89 ± 0.16[a]	3.2
Caspase-12	0.26 ± 0.06	0.79 ± 0.03[a]	3.0

Fold induction of mRNA was determined by scanning the pixel intensity of each band vs. the control level. GAPDH mRNA band was used to normalize the differences in loading. Data represent mean ± S.E. from six samples.

[a] $P < 0.05$ vs. control.

out with an antibody specific for cleaved caspase-9 to examine if DEX affected its processing. Fig. 3 shows that DEX treatment led to the conversion of the 49 kDa procaspase-9 to 39 and 37 kDa fragments.

Procaspase-11 was barely detectable in untreated cells. After DEX treatment, the two procaspase-11 bands (43 and 38 kDa) appeared along with a 30 kDa fragment. Caspase-12 was present as 60, 54 and 36 kDa bands in untreated cells. Upon DEX treatment, all of these bands were intensified (Fig. 3).

Poly(ADP-ribose) polymerase (PARP) is a substrate for caspases-3 [19], and cleaved PARP has been shown to be an important marker for apoptosis [20]. Lamin A is a substrate for caspase-6 and is essential for maintaining the integrity of nuclear envelope and cellular morphology [21]. Since both caspase-3 and caspase-6 were induced by DEX, cleaved PARP and lamin A levels were assessed in DEX-treated cells. As shown in Fig. 3, DEX treatment resulted in PARP and lamin A cleavage in a time-dependent manner, corresponding to the pattern of caspase-3 and caspase-6 activation.

Since caspase-3 is the most important effector caspase, we examined whether DEX induced caspase-3 enzyme

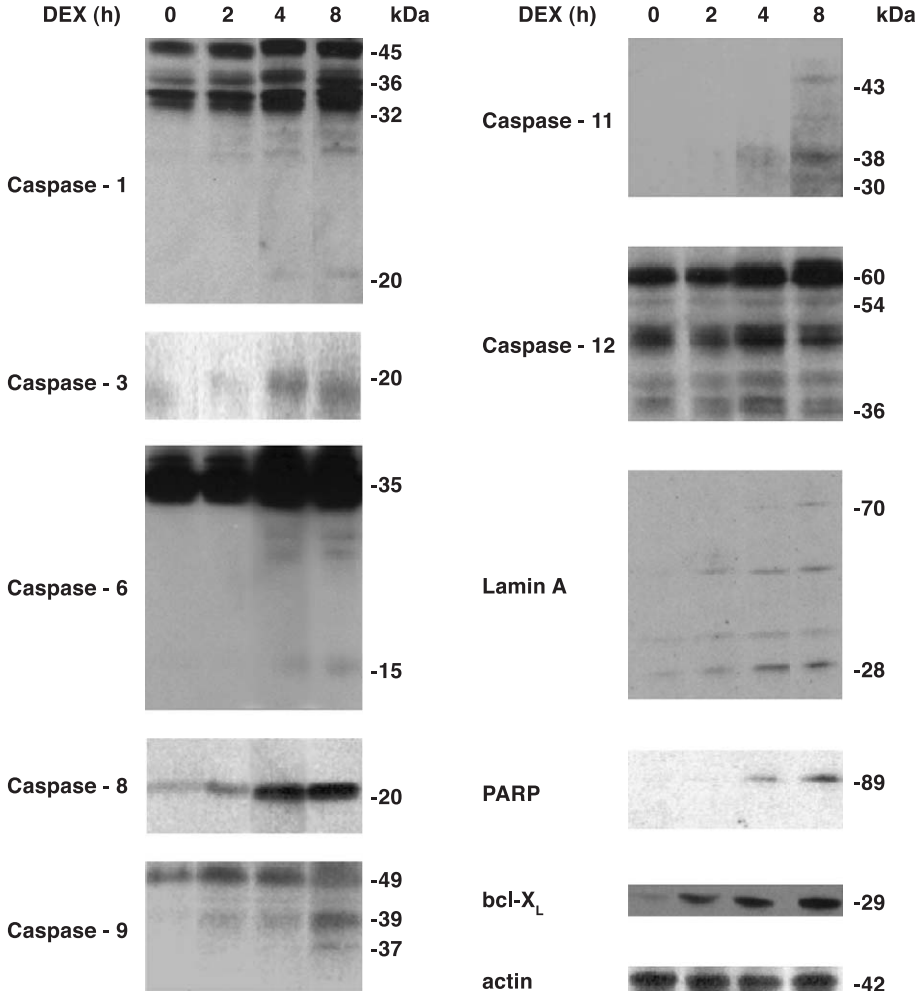

Fig. 3. Western blot analysis of caspases and bcl-X_L. MC3T3-E1 cells were treated with or without DEX for 2, 4, or 8 h. Western blot analysis was carried out with antibodies specific for caspases-1, -3, -6, -8, -9, -11, -12, cleaved PARP, lamin A, and actin. Antibody against actin demonstrates equal loading of proteins. Data represent three separate experiments.

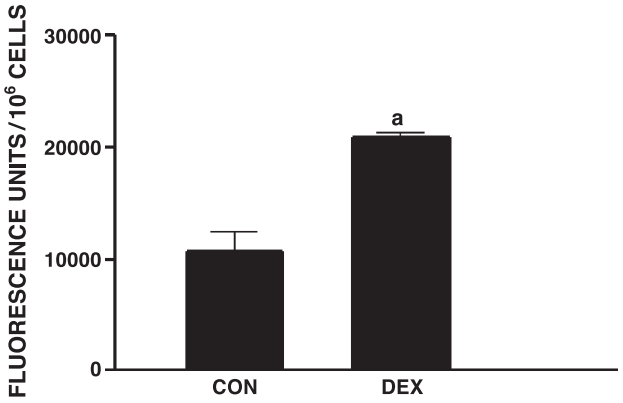

Fig. 4. Effects of DEX on caspase-3-like activity. MC3T3-E1 cells in 100 mm dishes were treated with DEX for 7 h. Caspase-3 activity was measured by a fluorochrome labeled caspase inhibitor binding assay as described in Materials and methods. Results were expressed as fluorescence units/10^6 cells. Data represent mean ± S.E. from four samples. a = $P < 0.05$ vs. CON.

activity. In this assay, a FAM-DEVD-FMK was used to bind to the active center of the activated enzyme [22]. Fig. 4 shows caspase-3-like activity was increased by 2-fold after 7 h of DEX treatment.

Cytochrome *c* release is a marker for mitochondria-related apoptosis [23]. Western blot analysis of cytochrome *c* was performed on cytosolic and mitochondrial fractions of control and DEX-treated cells. In control cells, most cytochrome *c* was found in the mitochondria (Fig. 5). After DEX treatment for 16 h, the amount of cytosolic cytochrome *c* was elevated, and the mitochondrial cytochrome *c* level was decreased. COX-4, a mitochondrial marker, remained at the same level.

Finally, DEX-induced apoptosis was detected by APOPercentage apoptosis kit, an assay that detects early apoptotic event. As shown in Fig. 6, control cells had very few red apoptotic cells. Upon DEX treatment for 3 h, approximately 15% of cells became apoptotic and stained red.

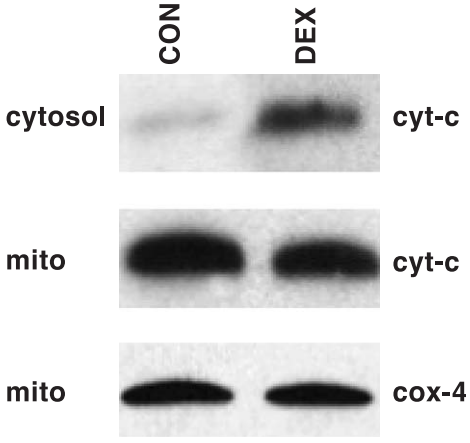

Fig. 5. DEX induced cytochrome *c* release into the cytoplasm. Mitochondrial and cytosolic fractions were prepared from cells treated with DEX for 16 h. Western blot analysis was carried out with cytochrome *c* and COX-4 antibodies.

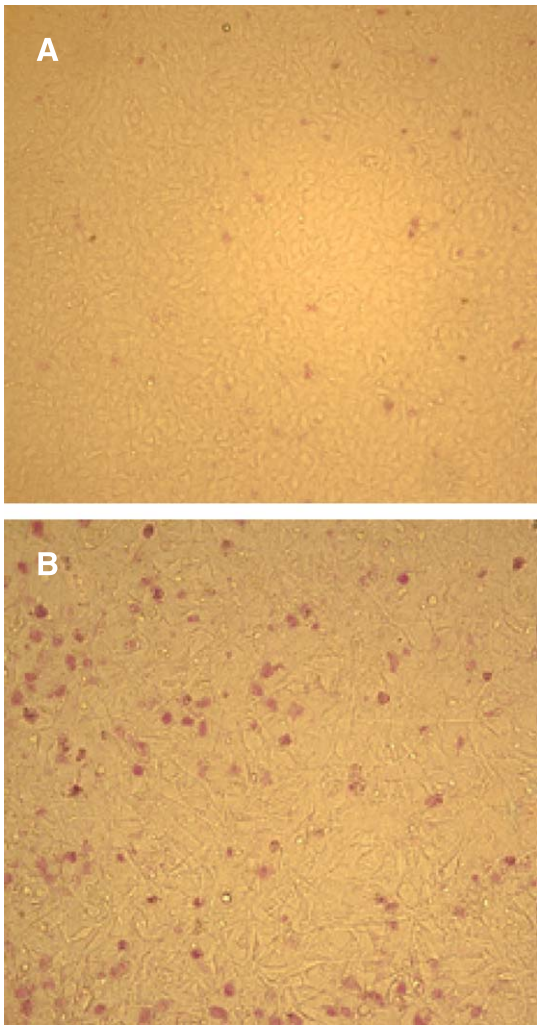

Fig. 6. Detection of apoptotic cells by staining with APOPercentage dye. Cells were treated with DEX for 3 h and stained with dye as described in Materials and methods. Apoptotic cells were stained red.

4. Discussion

In this study, we show that DEX induces apoptosis in murine osteoblastic MC3T3-E1 cells by activating caspases-1, -3, -6, -8, -9, -11, and -12. In addition, bcl-X_L was also upregulated. We are the first to describe the induction of caspases-1, -8, -9, -11, and -12 by DEX in osteoblasts. The specificity of induction is indicated by the observation that mRNA levels of caspases-2, -7, -10, and -14 and the other bcl-2 family were not altered by DEX.

Caspases, cysteine proteases with aspartate specificity, are important mediators of apoptosis. The activation of caspases arises from a number of mechanisms including transcriptional activation, regulation of IAP proteins,

autoactivation, or cleavage by other caspases [4,7]. Thus far, 14 members of the caspase family have been isolated. They can be categorized into three groups [6]: initiator caspases (-8, -9, -10, and -12), effector caspases (-3, -6, and -7), and inflammation caspases (-1, -11).

Caspase-8 is an initiator caspase that mediates signal transduction downstream of death receptors located on the membrane [24]. The induction of caspase-8 by DEX in osteoblasts agrees with the report of Schmidt et al. [25] that glucocorticoid-induced apoptosis in monocytes involved the CD95/CD95 ligand system, which led to the activation of both caspase-3 and caspase-8. Caspase-9 is another initiator caspase that is involved in mitochondrial damage [26]. In this study, DEX is shown to induce cytochrome *c* release in osteoblasts (Fig. 5). Cytochrome *c* is known to bind Apaf-1 and permit the recruitment of procaspase-9 [23].

Caspase-3 and caspase-6 are effector caspases that are responsible for cleaving nucleases in addition to cellular substrates including lamins and PARP [20,21]. Miyashita et al. [27] reported that glucocorticoid caused activation of caspase-6 but not caspase-3 in human pre-B leukemia 697 cells. In contrast, the present study indicates that both caspase-3 and caspase-6 are specifically upregulated by DEX in osteoblasts. The discrepancy between these studies may reflect cell specificity.

Caspases-1, -4, -5, -11, -12, and -13 belong to the caspase-1 subfamily. Our study shows that DEX upregulates three of the six members of the caspase-1 subfamily: caspases-1, -11, and -12. Caspase-1, which functions both as an initiator caspase and an effector caspase, is the best characterized enzyme in this subfamily and is known to play a major role in inflammation [28,29]. The upregulation of caspase-11 mRNA by DEX treatment (Fig. 1) supports previous studies that caspase-11 is crucial for apoptosis. Wang et al. [30] showed that caspase-11 knockout mice had impaired production of interleukin-1β and caspase-1 mediated apoptosis, indicating that caspase-11 is important for both inflammation and apoptosis. In addition to its role in activating caspase-1, caspase-11 also activates caspase-3 under pathological conditions [31].

We demonstrate for the first time that DEX upregulated caspase-12 in osteoblasts. Procaspase-12 is predominantly localized at the ER and is activated by ER stress such as disruption of ER calcium homeostasis or accumulation of excess protein in ER [32]. There has been evidence that DEX increases calcium influx in several cell systems [33–35]. The involvement of calcium in DEX-induced apoptosis of osteoblasts awaits further investigation.

It is interesting to note that DEX induces bcl-X_L, which is an anti-apoptotic protein [36]. The induced bcl-X_L may serve as a protective anti-apoptotic factor against future apoptotic stimuli. Alternatively, bcl-X_L may be cleaved by caspase-3, resulting in a potent apoptosis inducer, as reported by Clem et al. [37]. The exact role of bcl-X_L in DEX-treated osteoblasts remains to be determined.

Pereira et al. [38] reported that when primary fetal rat osteoblasts were exposed to 10^{-6} M cortisol in the presence of β-glycerophosphate, cell differentiation was delayed with a decrease in the percentage of apoptotic cells and an inhibition of caspase-3 activity. However, cortisol has no effect on apoptosis in the absence of β-glycerophosphate. In the present study, we demonstrate that DEX treatment led to increased caspase-3 activity and enhanced apoptosis in MC3T3-E1 cells, which are immortalized osteoblastic-like cells, resembling mature osteoblasts. These opposite results suggest that the effect of GC on osteoblasts is dependent on cell culture conditions and stage of differentiation.

In summary, we found that DEX induces apoptosis by upregulating caspases-1, -3, -6, -8, -9, -11, -12 in MC3T3-E1 cells. These results suggest that strategies such as caspase inhibitors may be developed for the treatment of glucocorticoid-induced osteoporosis.

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